ABUNDANCE, DIVERSITY, AND POTENTIAL CONTAMINATION SOURCES OF *ENTEROCOCCI* IN CREEKS

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ABUNDANCE, DIVERSITY, AND POTENTIAL

CONTAMINATION SOURCES OF *ENTEROCOCCI*

IN CREEKS

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FORMAT OF THESIS

This thesis is presented in a combination of *Science of the Total Environment* and formats outlined in the thesis manual by the Oklahoma State University graduate college. The use of this format allows the independent main chapters, Chapters III and IV, to be suitable for submission to scientific journals. Each main chapter is complete in itself with abstract, introduction, materials and methods, results, discussion, and reference section.

CHAPTER I

INTRODUCTION

Although 70% of the earth's surface is covered with water, 96% of water is in saline form and only 4% is in freshwater forms which include streams, creeks, rivers, lakes, and groundwater (USGS, 2009). Numerous studies showed that the quality and quantity of water resources are in grave threat from contaminations, such as fecal matters, radioactive materials, toxic metals, and toxic organic compounds.

Fecal matters may contain pathogenic and enteric microorganisms as well as therapeutic and subtherapeutic antibiotics and growth hormones (Harwood et al., 2000). Fecal microbes found in the environment include adenovirus, astrovirus, enterovirus and norovirus; *Salmonella* spp.; *Shigella* spp.; *Escherichia coli* (*E. coli*); *Crystosporidium* spp.; and *Enterococcus* spp. (Teixeira and Facklam, 2003). However, it is challenging to evaluate all potential fecal microbial contaminants when evaluating water quality. Instead, fecal indicator microbes, including fecal coliform, *E. coli* and *Enterococci*, were often evaluated because these microbes inhabit the intestinal track of warm blood animals.

They have also been positively correlated with microbial pathogens such as *Salmonella* and with the number of people being ill upon swimming the contaminated water (Cabelli et al., 1982; USEPA, 2004; Wade et al., 2006).

The needs for water quality criteria have been established regarding fecal microbial contamination due to mounting evidence of water contamination by fecal microbes. In the United States, a water body is considered impaired when fecal microbial counts exceed 126 CFU 100 mL^{-1} for *E. coli*, or 33 CFU 100 mL^{-1} for *Enterococci* in freshwater (USEPA, 2004). Currently 45% (387,218 miles) of rivers and streams and 59% (9,914,854 acres) of lakes, reservoirs and ponds in the US are impaired (USEPA, 2008). About 11% (98,074 miles) of rivers and streams and 3% (570,686 acres) of lakes, reservoirs and ponds are impaired by microbial contamination (USEPA, 2008). Microbial contamination of water is primarily due to non-point sources, which are challenging to address.

"Microbial Source Tracking" (MST) methods have been gaining increasing interest in addressing non-point source contaminations (Simpson et al., 2002). The challenges are to identify signature microbial species and establish a database for source tracking. Although, an effective source tracking method has yet to be developed. Identification of contamination sources still rely on observed correlations between microbial abundance and environmental variables.

In the effort of MST, the possibility of host-specificity in *Enterococci* is suggested (Soupir et al., 2006). Of 101 biochemical phenotypes evaluated, 10% of isolates were originated from human sources, 61% from animal sources, while

the rest from multiple hosts (Ahmed et al., 2005). Of the animal sources, 14% were unique to chickens, 7% to dogs and 6% to horses (Ahmed et al., 2005). Likewise, using antibiotic testing and discriminate function analysis, 72% of isolates were originated from municipal sources, livestock or wild birds (Ebdon and Taylor, 2006). The presence of *Enterococci* genogroups was found to be host specific (Bonten et al., 2001). Genogroups were found in pigs (genogroup A), poultry and poultry farmers (genogroup B), hospital patients (genogroup C), and veal calves and veal calf farmers (genogroup D) (Willems et al., 2000).

Water quality criteria differ based on different types of water bodies (USEPA, 2004). *Enterococcus* has been used as an indicator of fecal microbial contamination in the water environment (Ferguson et al., 2005; USEPA, 2004) including *Ent. faecalis* as human fecal indicator (Wheeler et al., 2002), however, understanding the diversity of *Enterococci* as affected by temporal and spatial variability would assist with evaluating responses of pathogens to different environmental conditions (Ferguson et al., 2005; Jeng et al., 2005; Molina, 2005; Turbow et al., 2003). *Enterococci* are used as microbial indicator in both fresh and marine water (USEPA, 2004). However, the species diversity is expected to change with the system (Ahmed and Katouli, 2008; Caugant et al., 1981). Thus, it is essential to determine environmental variables in the system of interest.

As conduits to many water bodies, creeks are important study sites for the evaluation of water contamination. Creeks are highly accessible to areas with both human and agricultural activities, and they are main conduits to larger water

bodies such as rivers, lakes and bays (Shanks et al., 2006; Traister and Anisfeld, 2006). Since many water bodies are impaired (13% of assessed miles of rivers and streams, 64% for acres of assessed lakes, reservoirs and ponds, and 38% of the square miles of bays and estuaries), source tracking is essential and should consider tributaries such as creeks as possible sources (USEPA, 2008).

We hypothesized that *Enterococci* abundance and diversity in the water environment vary in space and time, and their fluctuation in space and time may be linked to sources of contamination. Data obtained are presented in two main chapters, Chapters III and IV. Chapter III includes data and discussion on abundance and potential contamination sources in Stillwater Creek Watershed with a focus on detection, quantification, and evaluation of *Enterococcus* spp. in creeks with respect to temporal and spatial variations. Studies were also conducted to determine relationships between occurrence of *Enterococcus* spp. and environmental variables, and examine possible linkages between the occurrence of *Enterococci* and the multiple environmental factors. In Chapter IV, studies were focused on species diversity of *Enterococci* in creeks during baseflow and highflow conditions, and revealing whether diversity of *Enterococci* was linked to their potential contamination source. Further evaluations were conducted to assess *Enterococci* as an indicator for microbial source tracking.

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CHAPTER II

REVIEW OF LITERATURE

2.1. Water Contamination

Contaminations of water by fecal matters, radioactive materials, toxic metals, and toxic organic compounds are widespread, pose great health and environmental concerns, and thus, are receiving increasing attention. Identifying the source of contamination would assist developing effective management strategies to reduce or eliminate pollution. It is known that contaminations may be derived from point and non-point sources. Point sources are fixed facilities and specific individual or group that discharge contaminants directly into water bodies and are easily traceable (USEPA, 1977). A good example is a slaughter house that discharges effluent into a river without prior waste disposal treatment. On the other hand, non-point sources are sites or factors that pollute the water body but provide no definite mark about the pollutants' specific origin (USEPA, 1977). For example, manure applied onto agricultural land can be carried away during heavy rainfall into a stream. Oftentimes, non-point contamination is generally attributed to human activities such as agricultural production,

recreation, and wildlife-related activities. Of the two, contamination from non-point sources is more challenging to address.

Fecal matters include pathogenic and enteric microorganisms, growth hormones, and antibiotics (Harwood et al., 2000). Their specific origin is often untraceable. Known as potential health hazards, microorganisms in fecal matters pose great concern because they could continuously increase in number and diversity in the environment. Microbes in fecal matters include viruses such as adenovirus, astrovirus, enterovirus and norovirus; *Salmonella* spp.*; Shigella* spp.; *Escherichia coli (E. coli); Crystosporidium* spp.; and *Enterococcus* spp. (Weisberg, 2007; USEPA, 2004; Teixeira and Facklam, 2003). Relative to other fecal microorganisms, *Enterococcus* spp. are subsistent environmental contaminants that were not given much attention in the past. However, their existence in the environment is alarming due to their multiple drug resistance and their ability to acquire virulent genes (e.g. enterococcal surface protein gene) to become pathogenic (Bonten et al., 2001). More alarming though, as supported by evidence, these genes are bequeathed to other microorganisms making them pathogenic; and worse, pathogens may become multi-drug resistant (Bonten et al., 2001).

2.2. Characteristics of *Enterococci*

The genus *Enterococcus* belongs to the bacterial division Firmicutes, class Bacilli, order Lactobacillales and family Enterococcaceae (Brenner et al., 2005). This genus was not accepted until 1984, although it was proposed in 1970 (Teixeira and Facklam, 2003; Schleifer and Kilpper-bälz, 1984; Kalina, 1970). Before accepting as a genus, its members were commonly called “fecal streptococci” under the genus *Streptococcus*. They were treated as a special group because they manifested unique characteristics different from the rest of the *Streptococcus* spp. Unique to them is their intrinsic resistance to physical and chemical substances (Teixeira and Facklam, 2003). Generally, they are gram positive, facultative anaerobes, catalase-negative that occur as single, paired, or chained cocci (Brenner et al., 2005; Teixeira and Facklam, 2003). To isolate them into pure culture, their ability to hydrolyze esculin (e.g. in Enterocossel Medium) and their growth in a medium with 6.5% NaCl and 40% bile were proven to be useful (Teixeira and Facklam, 2003). This new genus was clearly defined from *Streptococcus* with the use of molecular tools such as DNA-DNA and DNA-rRNA hybridization techniques (Schleifer and Kilpper-Balz, 1984). Currently, there are 37 species classified under this genus (Carvalho et al., 2006; Svec et al., 2006; 2005a; 2005b; Brenner et al., 2005; Naser et al, 2005; Fortina et al., 2004; Koort et al, 2004; Vancanneyt et al., 2004; Teixeira and Facklam, 2003; Holt et al., 1994; Pompei et al., 1992).

2.3. Occurrence and diversity of *Enterococcus* in the environment

Enterococcus spp. are predominantly found in the gastrointestinal tract of humans and animals (Teixeira and Facklam, 2003). The most common species that are ordinarily isolated from human and animal feces is *Enterococcus faecalis*, while other species are found at lesser degrees (Teixeira and Facklam, 2003). They have also been isolated from plants, soil, and inanimate objects, which are attributed to fecal contamination (Kramer et al., 2006; Anderson et al., 2005; Jeng et al., 2005; Paulsen et al., 2003; Mundt, 1963a; 1963b). Although they have been studied for many years now, their classification and taxonomy have been frequently changing. Many aspects of their ecology remain unknown. However, since the late 1800, it is has been known that they are associated with many human diseases (MacCallum and Hastings, 1899).

Once upon a time, *Enterococcus* spp. or called *Enterococci* for short, were considered harmless bacteria that are normal part of the human gastro-intestinal tract. The first disease associated with *Enterococci* was reported in 1899. *Enterococcus faecalis* (then described as *Micrococcus zymogenes)* caused acute endocarditis and death (MacCallum and Hastings, 1899). Since then, more diseases were found to be associated with them which include urinary tract infection (UTI), endocarditis, meningitis, wound infection, and nosocomial bacteremia (Teixeira and Facklam, 2003; Kaye, 1982). Now we know that some of their species are pathogenic and detrimental to humans and animals. They

were reported to be detrimental to animals such as mice, guinea pigs, rabbits, dogs, pigs and cats causing diarrhea, anorexia, enteropathy, ascending cholangitis and ductal pancreatitis (Lapointe et al., 2000; Cheon and Chae, 1996; MacCallum and Hastings, 1899). *Enterococcus faecalis* and *Ent. faecium* are most commonly associated with human infections than any other *Enterococci* (Willems et al., 2005). Hospital records showed disease outbreaks due to their infection. From 1975 to 1984, urinary tract infections (UTI) caused by *Enterococci* increased from 6% to 16% (Morrison Jr. and Wenzel, 1986). By 1984, they were the second most frequently isolated pathogen in infection sites, next to *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)* (Morrison Jr. and Wenzel, 1986). Similar information was obtained in a two-year (Emori and Gaynes, 1993) and a thirteen-year hospital data (USHHS, 2004; 2000; 1999). Although *E. coli* and *S. aureus* were the most isolated nosocomial pathogens, the frequency and site of their isolation greatly differ. *Escherichia coli* was common in UTI sites and less common in other infection sites, whereas, *S. aureus* was rarely found in UTI sites but was frequent in other sites (Emori and Gaynes, 1993). However, *Enterococci* were commonly found in all infection sites (UTI, surgical infection and bloodstream infection) except respiratory tract (Emori and Gaynes, 1993). In addition, the 1990 to 2003 report from the U.S. Department of Health and Human Services (USHHS) indicated the concerning persistence of *Enterococci* in many hospital infections (USHHS, 2004; 2000; 1999). Although, the rate of increase in resistance (40 to 47% for 1999 to 2000

vs. previous 5 years) decreased to 12% in 2003 (vs. 1998 to 2004), the percent resistance of *Enterococci* appeared to increase from 24.7% in 1999 to 28.5% in 2003 (USHHS, 2004; 2000; 1999). Thus, it is obviously important to recognize *Enterococci* as great health risks to every individual.

The occurrence of hospital outbreaks due to *Enterococci* demanded reasonable explanation. It was suggested that irritation, adhesion and colonization of the bacteria in the brush borders of the small intestine result to disease such as diarrhea. This is due to the inhibition of the digestive enzyme which leads to poor digestion and absorption of the host (Cheon and Chae, 1996). Although, this mechanism was observed in pigs, it is an important information in disease development. In particular, this is essential because *Enterococci* do not manifest host selectivity (Laukova et al., 2004). Animal strains such as from horses, dogs, pigs, goats, chickens, cattle, lambs, rabbits and fish could adhere and colonize human hosts and vice versa at varying degrees (Heuer et al., 2006). In addition, *Enterococci* have been isolated not only from hospitalized patients but also from healthy and nonhospitalized individuals (Bonten et al., 2001). These individuals, both humans and animals, serve as reservoir or carriers (Willems et al., 2005).

Different isolates have been identified including the Vancomycin-resistant strains from healthy individuals (VRE) (Heuer et al., 2006). VRE is greatly associated with hospital outbreaks. In fact, *Enterococci* are long known to be intrinsically resistant to antibiotics. Yet, it is a concern because they manifest

resistance to leading antibiotics used for human and animal medication (Huycke et al., 1998). These antibiotics include cephalosporins, aminoglycosides, clindamycin, tetracyclines, erythromycin, avoparcin, teicoplanin and as earlier mentioned vancomycin (Laukova et al., 2004; Jett et al., 1994). However, the mere presence of antibiotic resistance does not alone lead to *Enterococci* causing disease. It was identified that the presence of virulence genes were likely responsible for disease development. These virulence genes express virulent factors for disease development, specifically, gelatinase, hemolysin, enterococcal adhesin (Ace), enterococcal surface protein (esp) and putative pathogenecity island (a cluster of virulence genes) (Vergis et al., 2002). Gelatinase degrades host's antibacterial peptides (Park et al, 2007; Schmidtchen et al, 2002). Hemolysin lyses the red blood cells (erythrocytes) (Chow et al., 1993; Ike, 1984). Enterococcal adhesion (Ace) is a mediating substance that intensifies cell to cell contact between bacterial cells and bacterial cell to eukaryotic cell (Jett et al., 1994; Kreft et al., 1992). Enterococcal surface protein (Esp) does not directly damage the hosts' cells but enhances the ability of the bacteria to colonize and persist in the host making the host susceptible for secondary infection (Shankar et al., 2001; 1999). Plethora of all these genes (both drug resistance and virulence) can be found in a single bacterial genome (Paulsen et al., 2003), thus, rendering *Enterococci* as highly adaptive microorganisms.

Transformation, transduction, conjugation and mutation are inherent to all bacteria. These characteristics allow them to easily adapt to their environment. These may also be the mechanism by which *Enterococci* developed as opportunistic pathogens. A study on *Ent. faecalis* revealed that more than one fourth of its genome consists of foreign or mobile DNA (Paulsen et al., 2003). *Amycolatopsis orientalis (Streptomyces orientalis), Streptomyces toyocaensis* and *Paenibacillus popilliae* are among the possible sources of genes (Bonten et al., 2001). Since *Enterococci* can accommodate high amount of foreign DNA, they are resistant in many physical, chemical and biological substances (Paulsen et al., 2003). In fact, sources of these foreign DNA are always available. The use and misuse of antibiotics result in resistance build-up and horizontal transfer of resistance genes in *Enterococci* (Bonten et al., 2001). Drug-resistant and virulent bacteria are not only found in hospital wastes but also in agricultural and urban wastes (Bonten et al., 2001). Improper waste disposal and agricultural run-off are just few of the reasons for bacterial contamination of the soil and water environment.

2.4. *Enterococcus* as a Microbial Indicator

Increasing human population and activity increase wastes disposed into the environment. This is evident in recreation and urban areas near creeks, river and beaches (Wade et al., 2006; Lamka et al, 1980; Varness et al., 1978). In

recreation areas, contamination is evident during summer, holidays and weekends (Varness et al., 1978). Septic tank failures, manure application, and runoff from animal grazing areas also contribute wastes into these water bodies (Soupir et al., 2006; Erkenbrecher Jr., 1981; Doran and Linn, 1979). Unfortunately, wastes disposed into the environment increase bacterial count in water, often associated with fecal matters. Since the human body has many bacterial entry points such as the eyes, ears, nose and mouth, humans can be susceptible to diseases caused by these bacteria (USEPA, 2004; 1976; Varness et al., 1978). Furthermore, as bacterial count increases, the risk of having a disease also increases. A recent study showed that a $\log_{10}$ increase in *Enterococcus* count results in increase of nearly 1.2 fold in the probability of gastrointestinal illness in swimmers (Wade et al., 2006). This correlation has been observed by many scientists then and the U.S. Environmental Protection Agency (USEPA) which resulted to the creation of the water quality criteria (USEPA, 2004; Cabelli et al., 1982).

The water quality criteria related to microbial contamination were published by the USEPA to inform the public about water safety and contamination level recommendations (USEPA, 2004). These criteria were a summary of studies conducted by early scientists and the agency itself (USEPA, 1986; 1976; Cabelli et al., 1982). Following a series of studies initiated in 1972 to identify the relationship of bacterial level to the number of swimmers that become ill after swimming, as well as, to determine the best indicator of pollution, the

agency recommended the use of fecal coliform as an indicator (USEPA, 1986; 1976). According to the recommendation, bacterial counts should not exceed a mean of 200 per 100 ml for a minimum of 5 samples, and 400 per 100 ml for each sample every day in a 30-day period. For fresh and marine water, on the other hand, the counts should not exceed a mean of 14 MPN (Most probable number) per 100 ml and 10% of the samples not exceed 43 MPN per 100 ml in shell-fishing water. These recommendations were revised in 1986 with the addition of *Escherichia coli* (*E. coli*) and *Enterococci*. The criteria added that "geometric mean of *E. coli* should not exceed 126 per 100 ml sample, and 33 per 100 ml sample for *Enterococci* in fresh water" (USEPA, 2004; 1986; 1976). Furthermore, *Enterococci* were added in marine water criterion indicating that their "geometric mean should not exceed 35 per 100 ml sample" (USEPA, 2004; 1986; 1976).

Fecal coliform, *E. coli* and *Enterococci* were observed to be effective indicators. Their presence suggests water degradation and health risk (Cabelli et al., 1982; USEPA, 1976). It was observed that their density is directly correlated to the density of microbial pathogens such as *Salmonella* (USEPA, 1986; 1976). If the density of these indicators exceeds the recommended level, there is a greater health risk for swimmers and other humans who come in contact with the contaminated water (Wade et al., 2006; Cabelli et al., 1982). Below these levels, acceptable rates of illness (8 in 1000 swimmers in fresh water and 19 in marine water) may still occur but at less risks (USEPA, 1986; 1976).

Enterococci were recommended as microbial indicator not only because of their correlation to microbial pathogens. They were also observed to adapt and survive in different environmental conditions and easily detected in contaminated water. They can survive in dry and inanimate surfaces for months (Kramer et al., 2006), in oligotrophic microcosm under the condition of starvation (Hartke et al., 1998), in sediments for at least a week (Anderson et al., 2005; Jeng et al., 2005), in planktons (Signoretto et al., 2004), and in macro-algae Cladophora (Byappanahalli et al., 2003) which can serve as secondary habitat (Ishii et al., 2006). They can even survive longer in an environment with less predators and more available nutrients (Desmarais et al., 2002). In addition, environmental condition such as seasonal change also affects *Enterococci's* survival. *Enterococcal* density was higher in late winter and early spring but the summer season record showed more cases of ailments in swimmers (Turbow et al., 2003). However, the relationship between the season, *Enterococcal* density and illness were not established. Furthermore, *Enterococci's* ability to settle into the bottom sediments enables them to survive for at least a week (Jeng et al., 2005). It was suggested that *Enterococci* are released during high water flow giving a high bacterial count (Jeng et al., 2005). However, an unpublished study conducted in our laboratory observed that 91% of IDEXX counts in the surface water during both the base-flow and high-flow sampling periods were *Enterococci*, while only 28% of those in the sediment samples were *Enterococci*. This suggests that the sediments are not the only source of *Enterococci* during

high-flow. It is clear though that a non-point source contributes these bacteria into the water environment. Since virulent *Enterococci* do exist in the environment and humans can be susceptible, it is now left to know what environmental factors will greatly favor *Enterococci's* persistence in the water environment.

2.5. Microbial Source Tracking with *Enterococci*

Concerns regarding water contamination have been addressed using different approaches, one of which is often coined as "Microbial Source Tracking Methods" or MST Methods (Weisberg et al., 2007). These methods aim to identify the source of contamination in order to assist watershed managers in implementing their best management strategies. These will minimize fecal inputs; thus, reduce public health risk from watersheds (Weisberg et al., 2007). Since remediation of contaminated sites is expensive, it is more cost-effective if sources of contamination are identified prior to remediation (Simpson et al., 2002). These include methods using gene or toxin identification, and direct measurement of microorganisms from fecal sources (Weisberg et al., 2007). Chemical methods such as detection of caffeine, urobilin, and coprostanol are also done (Piocos and dela Cruz, 2000). Molecular techniques such as ribotyping, polymerase chain reaction (PCR), gel electrophoresis, antibiotic

resistance, and biochemical techniques are the recent approaches (Weisberg et al., 2007).

With the use of modern MST methods, the possibility of host-specificity in *Enterococci* is suggested. Host specificity may be a link in determining the source of contamination (Soupir et al., 2006). A study evaluating 101 biochemical phenotypes indicated that 10% of isolates can be determined to be originating from human sources, 61% from animal sources, while the rest can be from multiple hosts (Ahmed et al., 2005). Of the animal sources, 14% are unique to chickens, 7% to dogs and 6% to horses (Ahmed et al., 2005). Likewise, using antibiotic testing and discriminant function analysis, 72% of isolates was effectively identified to originate from municipal sources, livestocks or wild birds (Ebdon and Taylor, 2006). Furthermore, the presence of genogroups in the genus *Enterococci* was found associated with specific host (Bonten et al, 2001). Genogroups were found in pigs (genogroup A), poultry and poultry farmers (genogroup B), hospital patients (genogroup C), and veal calves and veal calf farmers (genogroup D) (Bonten et al, 2001; Willems et al., 2000).

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CHAPTER III

ABUNDANCE AND POTENTIAL CONTAMINATION SOURCES OF *ENTEROCOCCI* IN STILLWATER CREEK WATERSHED

Abstract

Understanding variation of fecal indicator microorganisms over time and in space and factors that influence the variation is necessary in determining the sources and responses of fecal contaminants to physical and chemical changes in water environments. Using *Enterococci* as a microbial indicator of water quality, this study was conducted to determine temporal and spatial variations of *Enterococci* in Stillwater Creek Watershed and to investigate the interrelationships of environmental variables that influence these variations. *Enterococci* were detected and quantified in the surface water and sediments at eight locations (Sites 1 to 8) under four temporal conditions including baseflow June 2005, baseflow July 2008, baseflow November 2008, and highflow May 2008. The locations were selected using targeted sampling strategy to examine three potential sources, including intense animal and crop production,

urbanization, and intense wildlife and recreation activities. *Enterococci* concentration (log MPN 100 mL^{-1}) were computed from IDEXX Enterolert™ counts and the percentage of isolates confirmed as positive *Enterococci* (PPE) using a five-step confirmation procedure. Environmental factors such as pH, water temperature (ºC), water turbidity (A595), cumulative rainfall (mm) and water discharge (m^3 sec^{-1}) were evaluated and their influences to *Enterococci* concentrations were determined. With the exception of one site, *Enterococci* concentrations exceeded the U.S. Environmental Protection Agency (USEPA) recommended level in all other samples tested. Temporal variability depended on the flow conditions rather than the sampling season. The variation in *Enterococci* concentrations was primarily influenced by interrelated factors including turbidity, cumulative rainfall, and water discharge. Concentrations of *Enterococci* in the surface water during highflow period were hundreds times of those sampled during base-flow period. Therefore, effective stormwater management is critical in maintaining water quality in the environment. Not only was highflow a significant source of *Enterococci* to the water environments, but also considerable *Enterococci* persisted in the sediment, making sediments reservoir of *Enterococci* and source of potential contamination.

3.1. Introduction

Although 70% of the earth's surface is covered with water, 96% of water is in saline form and only 4% is in freshwater forms which include streams, creeks, rivers, lakes, and groundwater (USGS, 2009a). Numerous studies showed that the quality and quantity of water resources are in grave threat from contaminations, such as fecal matters, radioactive materials, toxic metals, and toxic organic compounds.

Fecal matters may contain pathogenic and enteric microorganisms as well as therapeutic and subtherapeutic antibiotics and growth hormones (Harwood et al., 2000). Fecal microbes found in the environment include adenovirus, astrovirus, enterovirus and norovirus; *Salmonella* spp.; *Shigella* spp.; *Escherichia coli* (*E. coli*); *Crystosporidium* spp.; and *Enterococcus* spp. (Teixeira and Facklam, 2003). However, it is challenging to evaluate all potential fecal microbial contaminants when evaluating water quality. Instead, fecal indicator microbes, including fecal coliform, *E. coli* and *Enterococci*, were often evaluated because these microbes inhabit the intestinal track of warm blood animals and were shown to be positively correlated with microbial pathogens such as *Salmonella* and with the number of people being ill when swimming in water bodies (Wade et al., 2006; USEPA, 2004; Cabelli et al., 1982).

The mounting evidence of water contamination by fecal microbes drove the needs to establish water quality criteria regarding fecal microbial

contamination. In the United States, a water body is considered impaired when fecal microbial counts exceed 126 CFU 100 mL^{-1} for *E. coli*, or 33 in freshwater and 35 CFU100 mL^{-1} in marine water for *Enterococci* (USEPA, 2004). Currently 45% (387,218 miles) of rivers and streams and 59% (9,914,854 acres) of lakes, reservoirs and ponds in the US are impaired (USEPA, 2008). About 11% (98,074 miles) of rivers and streams and 3% (570,686 acres) of lakes, reservoirs and ponds are impaired by microbial contamination (USEPA, 2008). Microbial contamination of water was primarily due to non-point sources, which are challenging to address.

"Microbial Source Tracking" (MST) methods have been gaining increasing interest in addressing non-point source contaminations (Simpson et al., 2002). The challenges are to identify signature microbial species and establish a database for source tracking. Unfortunately, an effective source tracking methods is yet to be developed. Attempt to identify contamination sources still rely on observed correlations between microbial abundance and environmental variables.

We hypothesized that *Enterococci* abundance in the water environment vary in space and time, and their fluctuation in time and space may be linked to the sources of contamination. The goal of this study was to detect, quantify, and evaluate *Enterococcus* spp. in creeks with respect to temporal and spatial variations, to determine relationships between occurrence of *Enterococcus* spp.

and environmental variable, and to examine possible linkages between the occurrence of *Enterococci* and the multiple environmental factors.

3.2. Materials and Methods

3.2.1. Study sites description

Eight sites were selected from the Stillwater Creek Watershed, which is located in central Oklahoma in the United States (Table 3.1, Figure 3.1). The study area covered approximately 3240 ha of land.

Sites were selected using targeted sampling strategy to evaluate the impact of animal and crop production (around Sites 2 and 3), wildlife and recreation (between Sites 8 and 5), and urbanization (between Sites 5 and 6) on microbial contaminations in the water environment. Site 1 was upstream, providing information before the creek water passed through areas of potential microbial contamination sources under evaluation. Site 7 was downstream, providing indications on survival and transport of fecal microbes in the environment.

The animal and crop production areas around Sites 2 and 3 covered about 1554 ha of land. Animal production areas included facilities for sheep (about 33 ha), dairy cattle (about 154 ha), beef cattle (about 16 ha), horses (about 24 ha), and swine (about 33 ha), (http://www.ansi.okstate.edu/facilities/). .

Table 3.1. Description of the sampling sites.

Sites	Location	Description
S1	36°7'48.90"N 97°8'25.00"W	Upstream of Stillwater creek, served as the control
S2	36°8'44.90"N 97°6'19.30"W	Cow creek, before passing through intense agricultural production areas
S3	36°6'57.00"N 97°5'55.70"W	Cow creek, after passing through intense agricultural production areas, the contribution of agricultural production was evaluated in this site
S4	36°6'25.30"N 97°5'22.70"W	Stillwater creek, water from Cow creek enters Stillwater creek, short-term survival and transportation of *Enterococci* were evaluated in this site
S5	36°8'15.80"N 97°3'41.10"W	Boomer creek, before passing through the city proper and after the Boomer lake, the contribution of wildlife and recreation to fecal contamination was evaluated in this site
S6	36°6'5.51"N 97°2'22.20"W	Boomer creek, after passing through the city proper, the contribution of urbanization to fecal contamination was evaluated in this site
S7	36°5'21.70"N 97°0'56.20"W	Downstream of Stillwater creek, the water from the three creeks merges at this site, prolonged survival and transportation of fecal contaminants were evaluated in this site
S8	36°9'35.74"N 97°3'40.59"W	Boomer creek, before passing through the Boomer lake which is a wildlife and recreation area

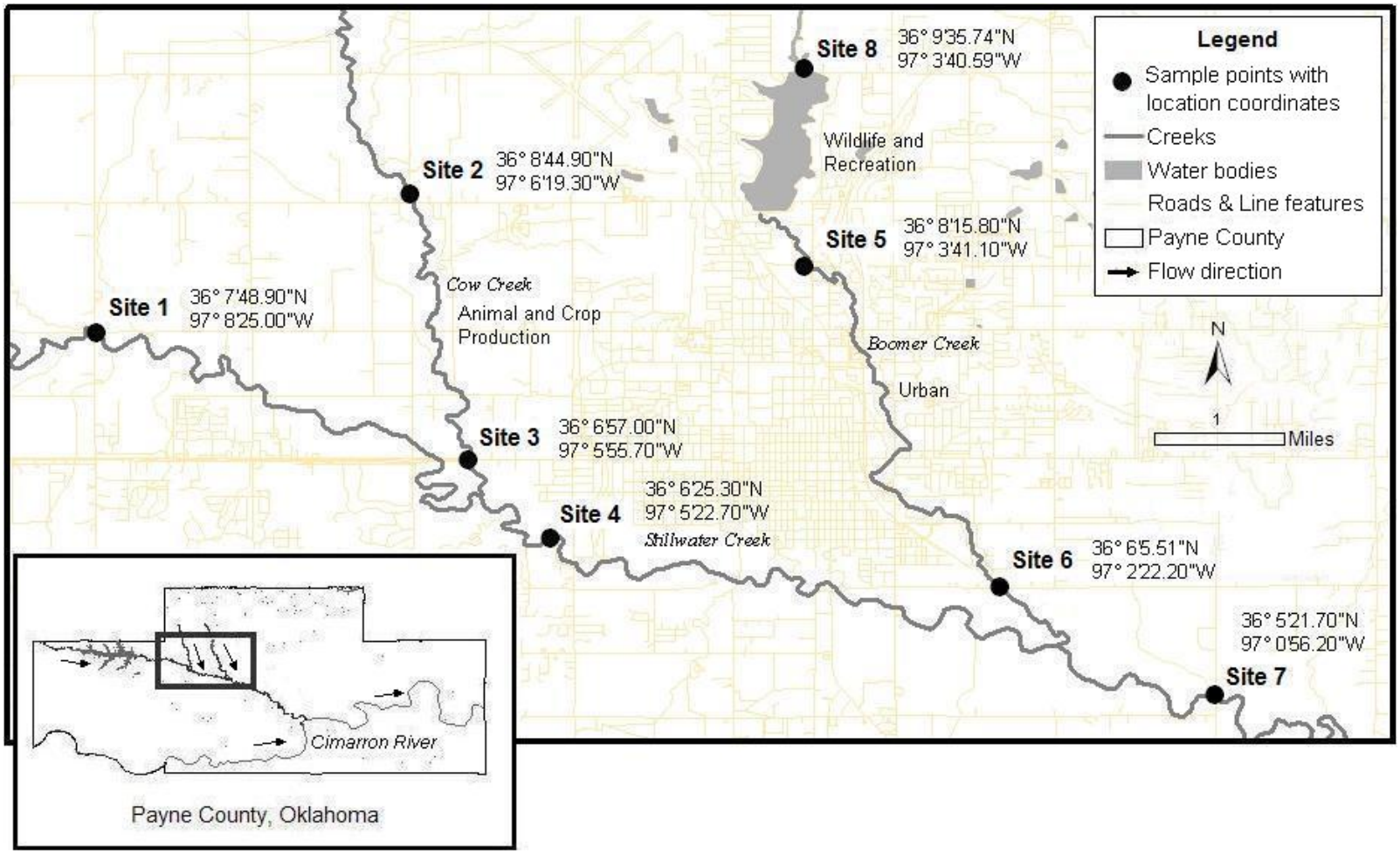

Figure 3.1. Location of sampling sites in Stillwater Creek Watershed. Potential contamination sources include: intense animal and crop production areas around Sites 2 and 3, intense wildlife and recreation area between Sites 8 and 5, and intense urbanization between Sites 5 and 6. Site 1, an upstream site, was used as a reference. Sites 4 and 7 were evaluated for transportation and survival of *Enterococci.*

At the time this study was conducted, there were at least 30 sheep, 90 dairy cattle, 215 cows and heifers, 980 beef cattle, 110 horses and approximately 1,000 heads of pigs in the facilities. The pigs were exclusively confined and were composed of 125 mature breeding animals and their offspring of various sizes. The 90 dairy cattle and 980 beef cattle were also confined. Other animals were grazed within the fenced facility. Each facility included confined stalls and houses. Animal waste produced were collected in a retention pond (equine and dairy cattle farm), a lagoon (beef cattle farm), a two-stage lagoon (swine and dairy cattle farms), or being treated in an anaerobic digester (swine farm) before being applied to land near the facilities. The first of the two-stage lagoon captured flushed waste water, and the second received overflow from the first lagoon which was subsequently land applied. Approximately 333,116 L of effluent from the swine facility was applied per day in each application that was done in 5 occasions targeting a four-mm deep spread over 8-ha area, which was used for Bermuda grass production (Personal communication). In general, 44 effluent applications were conducted per year over a growing season, with Bermuda hay production of 19 tons ha^{-1}. The forage production area in beef cattle farm (6 to 8 ha) is irrigated twice a year from a 1-ha lagoon leaving at least 275 cm deep of lagoon water without disturbing the sediments. The retention ponds and lagoons were approximately 240 to 1200 meters from Cow Creek.

The Boomer Lake located between Sites 8 and 5 is not only the home for about 450 resident geese (Zanin, 2008), and at least 650 birds of various species

and sizes, but also a popular recreational area for dog walking with about 50 to 100 dogs being walked in the area for 1 hr per day. On average, each goose generates at least 0.6 kg of waste per day (Fletcher, 2008), while a dog produces 0.34 kg of waste per day (Nemiroff and Patterson, 2007) that may be transferred to the lake by wind, rain, or other means. Water in Boomer Lake is discharged to Boomer Creek through an urban area, and then joins the Stillwater Creek. Residents between sampling Sites 5 and 6 all have sewer systems. However, house pets could be another source of fecal microbes to the creek. Approximately 400 houses are located along the Boomer creek between the two sites. An average household size in this city is two people (USCB, 2009), thus, a household with one pet would be about 800 people and 400 pets along this section of the Boomer creek as potential fecal microbial contamination sources.

3.2.2. Sampling

Surface water samples and sediments were collected in pre-sterilized containers (500-mL Nalgene® Bottles) during highflow and baseflow periods in different seasons and two different years.

Baseflow sampling was conducted when the cumulative precipitation for 10 days prior to sampling was less than 10 mm. Highflow condition is often referred to times when precipitation was more than 25 mm day $^{-1}$ (Gannon and Busse, 1989), and water discharge is more than the 75th percentile of normal flow

rate based on all daily data for the year (USGS, 2009b). The 75th percentile for this watershed is equivalent to 55 m^3 sec^{-1} (http://waterdata.usgs.gov/ok/nwis/nwis). In this study, highflow sampling was conducted when the cumulative precipitation in three consecutive days exceeded 50 mm. Based on data obtained by the USGS stream site station located in Cimarron River downstream of Stillwater creek, water discharge at the day of sampling was 200 m^3 sec^{-1} (Figure 3.2), which exceeded the 90th percentile (145 m^3 sec^{-1}).

Sampling under highflow condition was done in May 2008, while sampling under baseflow conditions were conducted in June 2005, July 2008 and November 2008. Rainfall and water discharge data 30 days prior to sampling were obtained from http://www.mesonet.org and http://waterdata.usgs.gov/ok/nwis/nwis, respectively (Figure 3.2), which did not only confer our definitions of baseflow and highflow, but also were also useful in data analysis and interpretation. Rainfall data presented were collected by Mesonet Station No. 89 located 2.0 miles west of Stillwater in Payne County, Central Oklahoma, U.S.A. at 36°7'15" N and 97°5'42" W with elevation of 272 meters above mean sea level, while, water discharge data presented was collected by USGS 07161450 Cimarron River near Ripley, Oklahoma Hydrologic Unit 11050003 Stream Site Station located in Payne County, Central Oklahoma, U.S.A. at 35°59'09" N and 96°54'43" W with drainage area of 17,979 square miles and datum of gage of 795.86 feet above sea level.

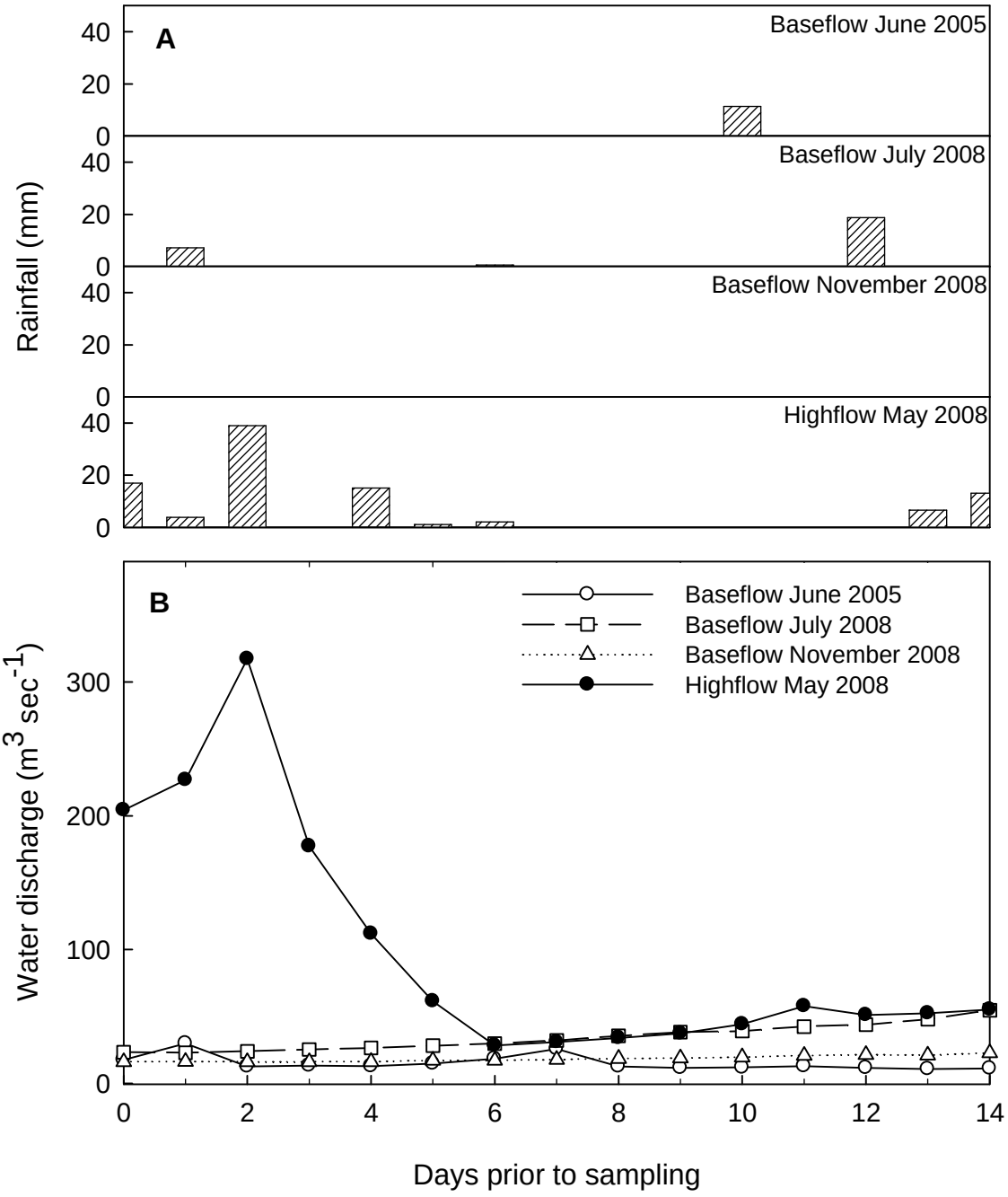

Figure 3.2. A) Rainfall events in mm, and B) water discharge in m^3 sec^{-1}, in Payne County, Central Oklahoma, U.S.A. from 14 days prior to sampling under baseflow conditions in June 2005, July 2008 and November 2008, and under highflow condition in May 2008 (sources: http://www.mesonet.org; and http://waterdata. usgs.gov/ok/nwis/nwis).

For each sample, approximately 100 mL surface water was obtained with a 500-mL pre-sterilized bottle. Sediment samples that were top organic matters in the stream bed were collected using a modified wide-mouth syringe (60 mL) and were placed in a 500-mL pre-sterilized bottle. All samples were placed on ice for transport, and were processed in the laboratory within hours after sampling. Only surface water was collected under highflow condition (May 2008) because creek water was very deep, while both surface water and sediments were collected under baseflow conditions (June 2005, July 2008 and November 2008). Three replicate samples were collected from each site for each sampling time. Seven sites were studied in 2005, while eight were evaluated in 2008. Therefore, a total of 162 samples were collected for the study with 93 surface water samples and 69 sediment samples.

3.2.3. Detection and quantification of *Enterococci*

IDEXX Enterolert^{TM} system (IDEXX Laboratories, Westbrook, ME) was used because it is more cost-effective and as effective as the other method (Budnick et al., 1996).

Surface water samples collected under baseflow condition were diluted to 10^{-1} and 10^{-2}, while surface water samples collected under highflow condition were diluted to 10^{-2} and 10^{-3} prior to detection by the IDEXX system. Sediment samples were shaken and were allowed to settle for an hour. The clear water on

the top layer of the sediments was removed using a sterile 10-mL pipette. Resettling the sediments and removal of the clear water on the top layer of the sediments procedure was repeated four more times. The remaining turbid layer was slowly poured into a new pre-sterilized 500-mL bottle, leaving behind any sand and other large particles that might create problems when sealing the Quanti-tray. The resulting samples were diluted to 10^{-2} and 10^{-3} using deionized water and processed similar to the surface water samples.

Enterococci from fluorescent (positive) wells were isolated and subjected to a five-step procedure to confirm them as positive *Enterococci*. One isolate was obtained from each well and about 25 isolates were evaluated for each sample. Total isolates evaluated for each sampling time ranged from 175 to 271 for surface water samples and 101 to 252 for sediments. The confirmation was based on their abilities to hydrolyze esculin and grow on brain heart infusion agar with 6.5% NaCl, being catalase negative, being gram positive and being ovoid or spherical under a phase-contrast microscope (McDonald et al., 2006; Kuntz et al., 2004; Wheeler et al., 2002).

The percentage positive *Enterococci* (PPE) of IDEXX most probable number (MPN 100 mL^{-1}) was used to calculate concentrations of *Enterococci* (MPN 100 mL^{-1} surface water or 100 g^{-1} sediments). Results for sediments were reported on dry-weight basis. Moisture content in the sediments was determined based on weight loss by drying the sample for 48 hours at 105ºC.

3.2.4. Measurement of other environmental factors

Surface water temperatures were recorded at the time of sampling. Water turbidity and pH were determined immediately following sampling using a spectrophotometer or pH meter. Rainfall and water discharge prior to sampling could be major factors affecting water quality in the area. Therefore, 30-day rainfall event (mm) and water discharge (m^3 sec^{-1}) prior to sampling under the four temporal conditions evaluated were obtained from Oklahoma mesonet database at http://www.mesonet.org and the US Geological Survey at http://waterdata.usgs.gov/ok/nwis/nwis, respectively (Figure 3.2).

3.2.5. Induction of Esculin hydrolysis ability for false positive isolates

IDEXX isolates that were not confirmed as *Enterococci* were considered false positive isolates. Forty-two false positive IDEXX isolates (24 from surface water and 18 from sediments) were cultured at 37ºC in Enterocossel broth in a 96 well microplate with 150 µl of broth in each well. After 24 hrs of growth, 10 µl of the microbial culture was transferred to a well that contained 140 µl of fresh Enterocossel broth in a sterile new microplate, and cultured at the conditions described above for another 24 hrs. All isolates were subjected to 12 daily subsequent transfers and culture to evaluate whether esculin hydrolysis of false positive isolates can be induced under conditions evaluated. This procedure

revealed gene expression ability of *Enterococci* in the environment which is essential in their detection and quantification.

3.2.6. Statistical procedures

Analysis of variance (ANOVA) and post-hoc mean comparison by least significant test (LSD, $p<0.05$) were conducted to evaluate differences in *Enterococci* concentrations among samples. Log transformations were conducted to meet the assumption of equal variance prior to ANOVA and post-hoc comparison (Freund and Wilson, 2003; Gomez and Gomez, 1984). For environmental samples involving multiple variables, principal component analysis (PCA) was conducted to reveal relationships among interrelated factors and to identify relative importance of the evaluated variables (Jolliffe, 1986). Because the evaluated variables were expressed in different units, PCA was performed using the correlation rather the covariance matrix (Jolliffe, 1986).

3.3. Results

3.3.1. Chemical and Physical Properties

Surface water temperatures ranged from 23 to 28ºC and 5 to 8ºC during the time of sampling in May to June and November, respectively. The pH values

of the surface water ranged from 7.4 to 8.0 (Table 3.2), while those of sediments ranged from 8.0 to 8.2. Turbidity of surface waters ranged from 0.0228 to 0.0514 for baseflow samples and 0.0605 to 0.4868 for highflow samples (Table 3.2). With the exception of Site 8, turbidity for surface water sampled under highflow was about 10-fold of those under baseflow.

3.3.2. *Enterococci* in surface water and sediments

3.3.2.1. Temporal Variation

The concentrations of *Enterococci* in surface water ranged from 2.05 to 2.62 log MPN 100 mL^{-1} under baseflow and 4.16 log MPN 100 mL^{-1} under highflow (Table 3.3). In the sediments, they ranged from 4.54 to 6.49 log MPN 100 mL^{-1}, about 100 fold higher than those in the surface water. The concentrations of *Enterococci* in the surface water under baseflow did not differ significantly regardless of the sampling year or month, but were about 100-fold lower than those under highflow. On the other hand, *Enterococci* concentrations in the sediments varied considerably between samples collected in 2005 and 2008 during different sampling periods. Their concentrations in sediments collected in July 2008 were about 100 fold higher than those collected in June 2005.

Table 3.2. pH and turbidity of the water samples taken from eight sites in Stillwater Creek Water under baseflow and highflow conditions.

Sites	pH		Turbidity (A595)	
	Baseflow	Highflow	Baseflow	Highflow
1	8.0	7.8	0.0276 ^{de}	0.2762 ^{f}
2	7.8	7.7	0.0373 ^{bc}	0.2959 ^{e}
3	7.8	7.4	0.0320 ^{cd}	0.4474 ^{b}
4	7.9	7.8	0.0375 ^{b}	0.4868 ^{a}
5	7.7	7.9	0.0514 ^{a}	0.0605 ^{g}
6	7.8	7.9	0.0228 ^{e}	0.3629 ^{d}
7	8.0	7.8	0.0372 ^{bc}	0.3735 ^{c}
8	8.0	7.7	0.0314 ^{d}	0.0656 ^{g}

Means followed by the same letter in a column are not significantly different from each other at 5% level of significance. Data include readings for samples taken under baseflow conditions in June 2005, July 2008 and November 2008, and under highflow condition in May 2008.

Table 3.3. Concentration (log MPN 100 mL^{-1} or g^{-1}) of *Enterococci* (*Ent.)* in surface water and sediments from eight study sites under four temporal conditions.[1]

	Temporal conditions[2]			
Sites[3]	Baseflow June '05[4]	Baseflow July '08	Baseflow November '08	Highflow May '08
	Ent. conc. in surface water			
	log MPN 100 mL^{-1}			
S1	2.38 cd	2.97 a	2.55 bc	4.51 ab
S2	2.67 bc	2.65 ab	1.72 d	4.32 ab
S3	3.33 a	2.21 c	2.91 a	5.06 a
S4	2.29 d	2.72 ab	2.43 c	4.77 ab
S5	2.87 b	2.85 a	1.51 e	4.05 b
S6	2.67 bc	2.45 bc	1.49 e	4.24 ab
S7	2.13 d	2.43 bc	2.66 b	3.93 b
S8	ND	0.00 d	1.16 f	2.43 c
Ave	2.62 C	2.28 C	2.05 C	4.16 B
	Ent. conc. in sediments			
	log MPN 100 g^{-1}			
S1	0.00 d	6.82 ab	5.11 b	ND
S2	4.22 c	6.34 bcd	5.08 b	ND
S3	6.18 a	6.13 d	5.39 ab	ND
S4	5.43 b	6.26 bcd	5.11 b	ND
S5	5.35 b	6.96 a	4.40 c	ND
S6	5.72 b	6.76 abc	4.38 c	ND
S7	4.90 c	6.45 abcd	5.66 a	ND
S8	ND	6.19 cd	5.76 a	ND
Ave	4.54 B	6.49 A	5.11 B	NA

[1] Means of three samples per site; Means followed by the same capital letter in a row or small letter in a column are not significantly different from each other within the row or column at 5% level of significance; ND, not determined, and NA, not applicable.

[2] Temporal conditions represented sampling under baseflow conditions in June 2005, July 2008 and November 2008, and under highflow condition in May 2008.

[3] Eight locations included Sites 1 to 8 (S1 to S8) which represented 3 potential contamination sources such as intense animal production (from Sites 2 to 3), intense wildlife and recreational activities (between Sites 8 to 5), and urbanization (between Sites 5 to 6).

[4] Source: C.T. Tan and S. Deng. 2005. Distribution and Occurrence of Indicator Fecal Bacteria in Stillwater Creek, Cow Creek, and Boomer Creek (Poster). Wentz Research Project.

3.3.2.2. Spatial variation

There were significant differences in *Enterococci* concentrations among sampling sites for both surface water and sediment samples (Table 3.3). The lowest concentration in surface water samples was generally found in Site 8, while the highest was found in Site 3. In sediments, their concentrations varied most for samples taken under baseflow in June 2005, but least for those taken under baseflow in July 2008. *Enterococci* concentrations at Site 2 ranged from 1.72 to 2.67 log MPN 100 mL^{-1}, which were generally lower than those at Site 3 (ranging from 2.21 to 3.33 log MPN 100 mL^{-1}). On the other hand, *Enterococci* concentrations in the sediments of Sites 2 and 3 within each sampling time were not significantly different.

The surface water and sediments collected under baseflow from Site 5 had higher *Enterococci* concentrations than those from Site 8. On the other hand, results for sediments in Sites 5 and 8 were inconsistent, with opposite trends observed for baseflow samples collected in July and November 2008.

Within each sampling time, there were little significant differences in *Enterococci* concentrations between Sites 5 and 6 in the surface water or sediments under baseflow or highflow (Table 3.3).

3.3.3. Percentages of IDEXX detection to be positive *Enterococci* (PPE)

In this study, esculin hydrolysis was a major selection step in identifying false positive of IDEXX detection. Some fluorescent wells from IDEXX Enterolert detection did produce bacterial isolates with the ability to hydrolyze esculin that turned the media to almost black color. Out of 350 cultures in fluorescent IDEXX wells evaluated in a preliminary study conducted, including 175 from surface water and 175 from sediments, 167 (95%) from surface water showed the ability to hydrolyze esculin, while only 60 (34%) from sediments showed the ability to hydrolyze esculin. Out of 167 isolates obtained from surface water that showed positive for esculin hydrolysis, 162 isolates were able to grow in Brain Heart Infusion that was supplemented with 6.5% NaCl. However, other confirmation steps, including catalase test, Gram staining, and morphology identification, did not select additional false positive out of the 165 isolates. For sediment samples, esculin hydrolysis was the only step that identified false positive from cultures in fluorescent IDEXX wells, while other confirmation steps used in this study did not select additional false positive out of 60 isolates that were positive for esculin hydrolysis.

Percentages of IDEXX detection to be positive *Enterococci* (PPE) in surface water were 99% for samples collected under baseflow in July 2008, 97% under highflow in July 2008, 91% under baseflow in June 2005, and 89% under baseflow in November 2008. In sediments, there were considerable variations in

PPE values at different temporal conditions. PPE was as low as 30%, and was as high as 93% (Table 3.4).

3.3.4. Induction of esculin hydrolysis in false positive *Enterococci* identified by IDEXX detection

IDEXX isolates that were not confirmed as *Enterococci* were considered false positive isolates. Out of forty-two false positive IDEXX isolates, 13 (31%) showed esculin hydrolysis after two subsequent transfers (Table 3.5). After 6 and 9 subsequent transfers, additional two isolates manifested esculin hydrolysis which resulted in a total 36% of isolates shown esculin hydrolysis ability.

Esculin hydrolysis was induced in 5 out of 24 isolates obtained from surface water samples (21%) and 10 out of 18 isolates obtained from sediments (56%).

3.3.5. Interrelationships of *Enterococci* concentration and other factors evaluated in the study

Significant correlations were observed among the variables evaluated with r values ranging from -0.28** to 0.83*** (Table 3.6). Variables tested included *Enterococci* concentration, PPE, pH, temperature, turbidity, 30-day cumulative rainfall prior to sampling, and water discharge at the day of sampling. The

Table 3.4. Percentage of IDEXX isolates confirmed as positive *Enterococci* (PPE) from surface water and sediments of eight sites under four temporal conditions.[1]

	PPE (%) from IDEXX isolates	
Temporal conditions	Surface water	Sediments
	91 [bcd]	30 [e]
Baseflow June 2005[2]	n=175	n=101
	99 [a]	93 [abc]
Baseflow July 2008	n=271	n=225
	89 [cd]	83 [d]
Baseflow November 2008	n=252	n=252
	97 [ab]	
Highflow May 2008	n=210	ND

[1] Mean percentages followed by the same letter in a row and a column are not significantly different from each other at 5% level of significance; ND = not determined; Isolates were subjected to a five-step confirmation procedure to identify positive *Enterococci* which are characterized by their ability to hydrolyze esculin, grow on brain heart infusion agar with 6.5% NaCl, show reaction as catalase negative and Gram positive, and being ovoid or spherical under a phase-contrast microscope (Wheeler et al., 2002; Kuntz et al., 2004; McDonald et al., 2006).

[2] Source: C.T. Tan and S. Deng. 2005. Distribution and Occurrence of Indicator Fecal Bacteria in Stillwater Creek, Cow Creek, and Boomer Creek (Poster). Wentz Research Project.

Table 3.5. Induction of esculin hydrolysis of 42 false positive IDEXX isolates by daily transfer and culturing in Enterocossel broth at 37°C.[1]

	Days of subsequent transfers in Enterocossel broth[2]											
Isolate description	1	2	3	4	5	6	7	8	9	10	11	12
Number of positive for esculin hydrolysis	0	13	13	13	13	14	14	14	15	15	15	15
% positive for esculin hydrolysis	0	31	31	31	31	33	33	33	36	36	36	36
Number of negative for esculin hydrolysis	42	29	29	29	28	28	28	27	27	27	27	27

[1] Isolates were positive for esculin hydrolysis when Enterocossel broth turned black within 24 hours at 37ºC.

[2] Glycoside esculin was hydrolyzed by *Enterococci* to form esculetin and dextrose. The medium contains iron salt and ferric ammonium citrate which react with esculetin resulting to blackening of the medium. To inhibit growth of other gram-positive and gram-negative bacteria, oxgall and sodium azide were present in the medium, respectively.

Table 3.6. Correlation coefficient (r) of *Enterococci* concentration, PPE and environmental variables measured in the surface water of eight sampling sites at four sampling times under baseflow (June 2005, July 2008 and November 2008) and highflow (May 2008) conditions (n=93).

	Enterococci concentration	PPE	pH	Temperature	Turbidity	Cumulative Rainfall
PPE	0.54***					
pH	-0.28**	-0.08				
Temperature	0.32**	0.21*	0.25*			
Turbidity	0.77***	0.22*	-0.45***	0.23*		
Cumulative Rainfall	0.54***	0.23*	-0.21*	0.81***	0.56***	
Water discharge	0.73***	0.54***	-0.50***	0.29**	0.83***	0.68***

Significance levels of correlations: * p< 0.05; ** p< 0.01; *** p < 0.001; PPE = percentages of IDEXX detection to be positive *Enterococci*; Cumulative rainfall (mm) of 30 days prior to sampling; Water discharge rate (m^3 sec^{-1}) at the day of sampling.

interrelationships among variables were evaluated by principal component analysis (PCA). The results are expressed as principal components 1, 2 and 3 (PC1, PC2 and PC3) of the total variances (Table 3.7). PC1 accounted for 52% of the total variance which was mostly attributed by *Enterococci* concentration, turbidity, cumulative rainfall and water discharge. PC2 accounted for 21% and was contributed mostly by pH and temperature. PC3 accounted for 14% and was contributed mostly by PPE and *Enterococci* concentration. *Enterococci* concentration, turbidity, cumulative rainfall and water discharge, were positively correlated with PC1, suggesting these variables were also positively correlated with each other. There were some positive relationship between PPE and *Enterococci* concentration.

The principal scores of the variables evaluated (PC1 and PC2) were plotted against sampling times under different temporal conditions (Figure 3.3). The separation of data was mainly due to PC1, with very little differentiation among the temporal conditions in terms of PC2. Distinct clusters were shown by sampling times under highflow in May 2008, baseflow in November 2008, and a cluster for the baseflow in June 2005 and July 2008.

Table 3.7. Principal component loadings of variables evaluated.

	PC1	PC2	PC3
Eigenvalue	3.64	1.48	1.00
Explained variance (%)	52	21	14
Enterococci concentration	0.45	-0.06	0.31
PPE[1]	0.23	0.10	0.85
pH	-0.23	0.60	0.13
Temperature	0.27	0.66	-0.17
Turbidity	0.45	-0.22	-0.09
Cumulative Rainfall[2]	0.43	0.33	-0.28
Water discharge[3]	0.47	-0.18	-0.21

[1] PPE = percentages of IDEXX detection to be positive *Enterococci*

[2] Cumulative rainfall from 30 days prior to sampling.

[3] Water discharge at the day of sampling.

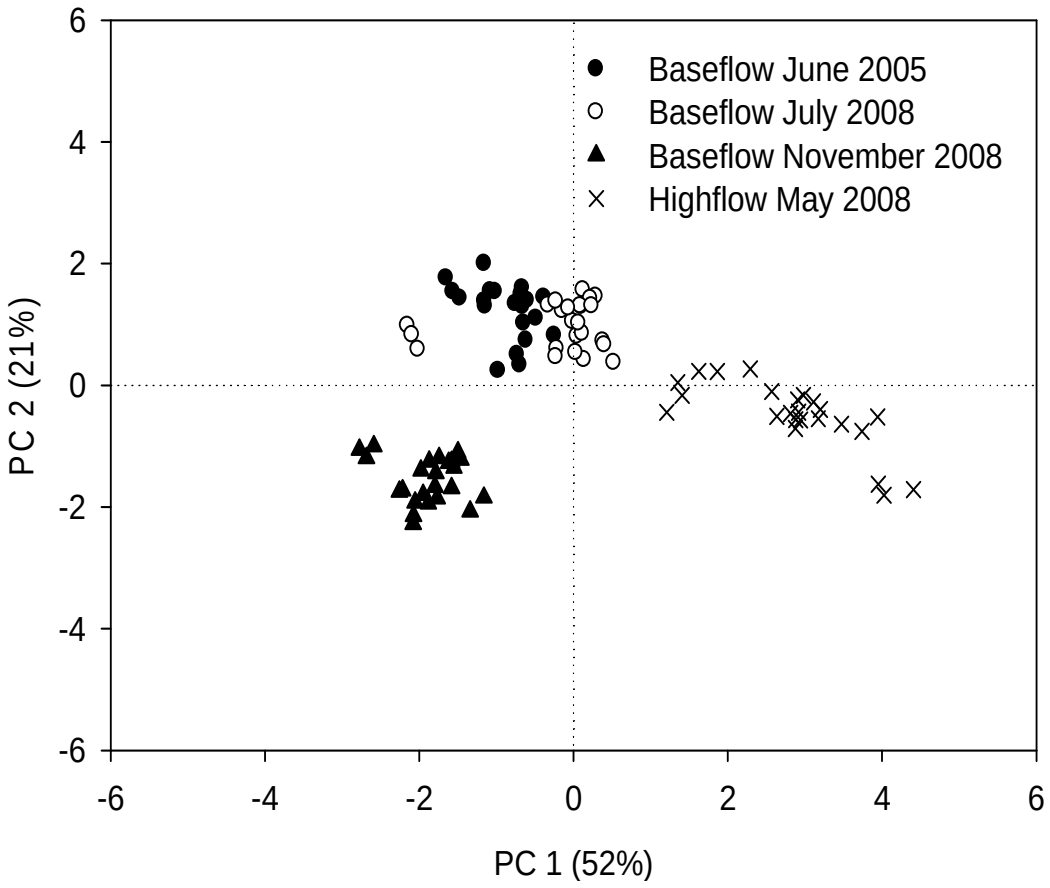

Figure 3.3. Principal component scores of evaluated variables against sampling times under four temporal conditions.

3.4. Discussion

The level of microbial indicators in the surface water and sediments of a watershed denotes probable quality of the water system. A water body that is in good condition meets its intended use and sustains life of aquatic flora and fauna. A high level of microbial indicators which is greater than the recommended rate by USEPA connotes high environmental and human health risk. A freshwater environment is considered contaminated when the concentration of *Enterococci* exceeds 33 CFU 100 mL^{-1} or 1.53 log MPN 100 mL^{-1} (USEPA, 2004). In this study, most samples tested exceeded this level, with the exception of surface waters sampled from Site 8.

Enterococci present in the water environments are either recently introduced or were introduced in the past but persisted in the environment. It is not clear what the dominant sources were for the watershed evaluated. The observed high levels of *Enterococci* under highflow over baseflow with 100-fold difference suggested the introduction of fecal microbes into the water system by highflow conditions, which is consistent with those reported by several other studies (Traister and Anisfeld, 2006; Gannon and Busse, 1989). This implies that effective management of creekwater during highflow events is critical in maintaining water quality in the environment. Further, changes in fecal indicator concentrations were limited under normal flow conditions (This study, Cabral and Marques, 2006; Davis et al., 2005) ranging from 0 to 3 log MPN 100 mL^{-1} (this study) and even higher from 3 to 5 log MPN 100 mL^{-1} (Cabral and Marques,

2006). These results suggest that recently introduced *Enterococci* could be a significant part of the detected fecal microbial community. On the other hand, the high levels of *Enterococci* in the sediment which was approximately 3 to 7 log MPN 100 g^{-1} (this study; Feng et al., 2007) is 100-fold of those in the surface water indicating that introduced *Enterococci* could persist in the environment. Studies by Hartel (2005) showed that sediment could serve as a reservoir for fecal microbes in the water system.

Agricultural production, wildlife and recreation activities, and urbanization can potentially contribute fecal microorganisms in water environments (Hubbard et al., 2004; Harwood et al., 2000). Concentrations of *Enterococci* were significantly higher in the creek water that passed through agricultural production facilities and wildlife and recreation area suggesting the possible impact of agricultural production, wildlife, and recreation activities on fecal bacterial contamination in watersheds. The impacts of animal and crop production on *Ent.* concentrations were inconsistent among samples from different temporal conditions, while the impacts from urbanization were not clear. Wildlife animals residing nearby water bodies are obvious sources of fecal microorganisms because they have direct access to the water bodies (Kuntz et al., 2004; Harwood et al., 2000), while, domestic animals that are confined or fenced indirectly introduce fecal microorganisms into the water environment through run-off or erosion of soil from grazing and effluent-irrigated lands (Toze, 2006; Hubbard et al., 2004).

The abundance of fecal microorganisms in water environments is influenced by several and interrelated environmental factors. Rainfall and water discharge are among the major players that influenced *Enterococci* concentration (This study; Jeng et al., 2005). In addition to the contributing sources, survival and persistence of fecal microbes in the environment are affected by other major factors such as temperature, pH, and turbidity. These factors had been linked to transport and persistence of bacteria in the water system (McDonald et al., 2006; Traister and Anisfeld, 2006; Crump et al., 1998). Fecal microorganisms attach to particles which are transported by water run-off during heavy rain and rapid water discharge (Jeng et al., 2005; Crump et a., 1998). *Enterococci* may attach not only to sediment particles but also other particles present in the water such as phytoplanktons (Signoretto et al., 2004). Aside from attachment to particles, *Enterococci* persist and re-grow in the water environment as long as nutrients from fecal sources are available (Byappanahalli et al., 2003; Desmarais et al., 2002). Temperature was also reported to affect the concentration of fecal microorganisms including *Enterococci* in water due to their significant positive correlation (This study; Koirala et al., 2008; Traister and Anisfeld, 2006). It is suggested, though, that temperature has limited effect on the level of *Enterococci* in water when their concentrations were observed on seasonal basis (This study; Cabral and Marques, 2006). This was confirmed in this study in that there were little relationship between *Enterococci* concentration and temperature based on PCA. It is possible that in days of low temperature, animal hosts are less mobile

making introduction of fecal microorganisms in water environment limited (Cabral and Marques, 2006).

Enterococci concentration and PPE also showed positive correlation and belonged to the same principal component group, indicating their close relationship. Since esculin hydrolysis (major step for confirming true positive *Enterococci*) was successfully induced in this study, it is suggested that fecal microorganisms have the ability to either turn on or off the gene(s) responsible for esculin hydrolysis, such as the gene for ß-glucosidase enzyme production (Hawksworth et al., 1971; Swan, 1954). As a result, *Enterococci* may become undetectable in the water environment. Gene expression in *Enterococci* had been previously reported related to other functions such as those involved in pathogenicity and antibiotic-resistance (Haack et al., 2009; Cho and Caparon, 2006; Harwood et al., 2000).

3.5. Conclusions

Concentrations of *Enterococci* in the surface water during highflow period were approximately 100 times of those sampled during base-flow period. Therefore, effective storm water management is critical in maintaining water quality in the environment. Not only was highflow a significant source of *Enterococci* to the water environments, but also considerable *Enterococci* persisted in the sediment, making sediment a reservoir of *Enterococci* and a source of contamination.

The impacts of animal and crop production on *Ent.* concentrations were inconsistent among samples from different temporal conditions, while the impacts from urbanization were not clear. This indicates that examining potential sources of contamination and pinpointing specific contamination sources are challenging which may not only require analysis of abundance data but also establishing effects of other environmental variables.

Esculin hydrolysis was a major selection step in confirming positive *Enterococci*. Some so-called false positive *Enterococci* detected by IDEXX reagents but not confirmed by USEPA recommended procedures might be true *Enterococci* without expressing genes responsible for esculin hydrolysis, which was evidenced by the induction of esculin hydrolysis in false positive isolates. A higher percentage (56%) induction among false positive isolates from sediments than those from surface water (21%) were observed. For all 1484 isolates evaluated, PPE in the surface water was high (91 to 99%), while wide variation (30 to 93%) observed for sediments. In addition, PCA indicated that PPE and *Enterococci* concentration was correlated. These suggested that genes responsible for esculin hydrolysis in *Enterococci* was not expressed constitutively once the organism was released to the environment, but could be induced at 37^{o}C in the presence of the substrate, glycoside esculin. Thus, in the evaluation of *Enterococci* in the environment, caution should be exercised in detection methods and data interpretation, and further study may be needed to evaluate the *Enterococci* confirmation steps currently used.

3.6. References

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CHAPTER IV

DIVERSITY OF *ENTEROCOCCUS* IN CREEKS

Abstract

Enterococcus species was reported to be presumably host specific making it an ideal microbial indicator for bacterial source tracking. In this study, the occurrence and diversity of *Enterococcus* species were investigated to determine if they can be linked to sources of contamination in Stillwater Creek Watershed. *Enterococci* isolated from surface water and sediments of eight sampling sites in Stillwater Creek Watershed collected during base flow and high flow (surface water only) conditions of summer 2005 and summer 2008 were subjected to genotypic characterization using polymerase chain reaction (PCR). Two sets of primers (Ent-1 and Ent-2, and ITS-RBS L1 and L2) were used to confirm isolates as *Enterococci*, and identify their species groups, respectively. Dice Similarity Coefficient was used to define similarity of the isolates from ATCC known references. The similarity coefficient values ranged from 1 to 0, with 1 showing the closest similarity. Seven species group were found with 4 groups (Groups A, B, C, and D) common in all the sites and three (Groups E, F, and G)

showing some site specificity. These groups represented six *Enterococcus* species including *Ent. faecalis* (Groups A and B), *Ent. faecium* (Group C), *Ent. gallinarum* (Group D), *Ent. hirae* (Group E), *Ent. avium* (Group F) and *Ent. dispar* (Group G). *Enterococcus faecalis* was the most dominant species composed of 68.3% of the 702 isolates evaluated. Although some studies suggested that *Enterococcus* sp. such as *Ent. faecalis* shows host specificity rendering it ideal indicator for microbial source tracking, this study suggests otherwise for common and dominant species such as *Ent. faecalis, Ent. faecium* and *Ent. gallinarum*, while, the less dominant species including *Ent. avium, Ent. hirae* and *Ent. dispar*, composed of 0.4 to 3.1% of the detected *Enterococci*, may be more useful in microbial source tracking.

4.1. Introduction

Enterococcus has been used as an indicator of fecal microbial contamination in the water environment (USEPA, 2004; Ferguson et al., 2005; Molina, 2005). A water body with high level of *Enterococci* is rendered unsuitable for its intended use (USEPA, 2004; Simpson et al., 2002). Due mainly to high level of indicator microbes, 10,016 miles of rivers and streams in 2008 were threatened and impaired (USEPA, 2008).

Microbial indicators and pathogens are directly related (USEPA, 2004), thus, understanding the diversity of *Enterococci* as affected by temporal and

spatial variability would assist understanding responses of pathogens to different environmental conditions (Ferguson et al., 2005; Jeng et al., 2005; Molina, 2005; Turbow et al., 2003). This is essential in developing strategies to predict contamination levels based on past and current environmental changes (Martin et al., 2007; Weisberg et al., 2007; Simpson et al., 2002) in an attempt to determine health risks of recreational and watershed areas.

Enterococci diversity can be linked to sources of contamination (Soupir et al., 2006; Ferguson et al., 2005). With the use of different approaches, such as determination of a particular gene (enterococcal surface protein) or toxin, direct isolation in selective medium, assessment of specific chemical component, and several molecular techniques, specific species of *Enterococci* and their occurrences can be determined (Weisberg et al., 2007; Piocos and dela Cruz, 2000). Molecular techniques such as ribotyping (Ferguson et al.) and antibiotic resistance have been employed (Weisberg et al., 2007; Tyrrell et al., 1997; Jensen et al., 1993).

In the effort of microbial source tracking (MST), the possibility of host-specificity in *Enterococci* is suggested, where, host specificity may be a link in determining the source of contamination (Soupir et al., 2006). A study evaluating 101 biochemical phenotypes indicated that 10% of isolates can be determined to be originating from human sources, 61% from animal sources, while the rest can be from multiple hosts (Ahmed et al., 2005). Of the animal sources, 14% are unique to chickens, 7% to dogs and 6% to horses (Ahmed et al., 2005).

Likewise, using antibiotic testing and discriminate function analysis, 72% of isolates was effectively identified to originate from municipal sources, livestocks or wild birds (Ebdon and Taylor, 2006). Furthermore, the presence of genogroups in the genus *Enterococci* was found associated with specific host (Bonten et al., 2001). Genogroups were found in pigs (genogroup A), poultry and poultry farmers (genogroup B), hospital patients (genogroup C), and veal calves and veal calf farmers (genogroup D) (Bonten et al., 2001; Willems et al., 2005).

Thus, the goal of this study was to detect and evaluate species diversity of *Enterococci* in creeks during baseflow and highflow conditions. We hypothesized that *Enterococci* diversity in the water environment were directly linked to their potential sources, and varied in space and time. Specifically, the objectives were to reveal the diversity of *Enterococci* in the environment; to examine whether diversity of *Enterococci* is linked to their potential contamination source, and to assess *Enterococci* as an indicator for microbial source tracking.

4.2. Materials and Methods

4.2.1. Study sites description

As described in Chapter III, eight sites were selected from the Stillwater Creek Watershed, which is located in central Oklahoma in the United States (Figure 3.1). The study area covered approximately 3240 ha of land. Detailed

site description is reported in Chapter III. Briefly, sites were selected using targeted sampling strategy to evaluate the impact of animal and crop production (around Sites 2 and 3), wildlife and recreation (between Sites 8 and 5), and urbanization (between Sites 5 and 6) on microbial contaminations in the water environment. Site 1 was upstream, providing information before the creek water passed through areas of potential microbial contamination sources under evaluation. Site 7 was downstream, providing indications on survival and transport of fecal microbes in the environment. The animal and crop production areas around Sites 2 and 3 covered about 1554 ha of land. Animal production areas included facilities for sheep (about 33 ha), dairy cattle (about 154 ha), beef cattle (about 16 ha), horses (about 24 ha), and swine (about 33 ha), (http://www.ansi.okstate.edu/facilities/).

4.2.2. Species Identification of *Enterococci* by Polymerase Chain Reaction

A total of 702 *Enterococci* isolates were evaluated for genetic diversity. These isolates were obtained from the surface water and sediments of the eight sampling sites taken during the base flow and high flow conditions in summer 2005 and summer 2008 and were subsequently verified by physiochemical and morphological means, including Enterococcosel agar plates (black colonies) and brain heart infusion agar (BHIA) plates supplemented with 6.5% NaCl. Positive isolates were further confirmed to be catalase negative, gram positive, and

shown to be spherical or ovoid cells under a phase contrast microscope. These isolates were stored at -80^{o}C for use.

Enterococcosel isolates were recovered and cultured for 12 hours at 35^{o}C in fresh brain heart infusion broth supplemented with 6.5% NaCl. Fifty μL of the culture was place in a 1.6 mL-eppendorf tube, which was then placed at 94ºC for 10 min (PCR thermocyclers was used), followed by -80ºC for 10 min to lyse the bacterial cells. The lysed cell culture was subsequently centrifuged at 10,000 rpm for 5 min to separate the DNA in the supernatant from other bacterial cell components in the precipitate. The supernatant was transferred to a fresh eppendorf® tube, which was used as DNA templates for subsequent polymerase chain reactions (PCR).

Three hundred seventy (370) of these isolates were further confirmed as *Enterococci* using a set of primers (Ent-1 and Ent-2) that are presumably specific only to *Enterococci*. The primer sequences are: Ent-1, 5'-TAC TGA CAA ACC ATT CAT GAT G-3', and Ent-2, 5'-AAC TTC GTC ACC AAC GCG AAC -3'. Positive *Enterococci* characterized by positive production of bands in 0.8% agarose gel were counted. A total of 370 isolates were tested and 359 (97%) showed positive production of bands.

Species diversity was evaluated by PCR amplification of the intergenic spacer (ITS-PCR) between the 16S and 23S rRNA genes using primers used ITS-RBS L1, 5'-CAAGGCATCCACCGT-3', and L2 5'-GAAGTCGTAACAAGG-3' (Tyrrell et al., 1997; Jensen et al., 1993). The PCR amplicons were digested with

SaU3AI restriction endonuclease enzyme and DNA bands were separated by electrophoresis in 0.8% Polyacrylamide gel to generate banding patterns that were shown to be species specific (Tyrrell et al., 1997).

The number of band and band mobility (position) were used to group the isolates, and the resulting banding patterns were compared with banding patterns of known standards for identification of the species. Standards used included *Ent. faecalis* ATCC 19433, *Ent. faecium* ATCC 19434, *Ent. gallinarum* ATCC 49573, *Ent. dispar* ATCC 51266, *Ent. sulfureus* ATCC 49903, *Ent. avium* ATCC 14025 and *Ent. hirae* ATCC 8043. Dice Similarity Coefficient was computed to define similarity of the isolates from these standards (Merquior et al., 1994; Dice, 1945). Formula for similarity coefficient is Dice= 2C/ N1 + N2, where C is the number of positive band matches, N1 is the total number of bands in the standard, and N2 is the total number of bands in the sample. A similarity coefficient value of 1 indicates that the sample and the standard are similar species, while a value of 0 indicates they are least similar.

4.2.3. Statistical procedures

Analysis of variance (ANOVA, $p<0.05$) was conducted for *Enterococci* percentages data. Prior to ANOVA, arcsine transformation was conducted to meet the assumption of equal variance prior to ANOVA (Freund and Wilson,

2003; Gomez and Gomez, 1984). The standard errors were computed using the arcsine transformed values (Freund and Wilson, 2003).

4.3. Results

4.3.1. DNA Profile of *Enterococci* species isolated in surface water and sediments in Stillwater Creek Watershed

Banding patterns of representative isolates for the seven *Enterococci* groups are shown in Figure 4.1. Of the 702 isolates evaluated, seven distinct patterns were exhibited and were grouped as species A, B, C, D, E, F, or G. Undigested PCR products yielded amplicons with sizes from 300 to 600 bp. Digested PCR products showed three to five major bands varying in size from approximately 140 to 300 bp. These patterns were compared with known ATCC standards described above.

Based on Dice similarity coefficients, species A and C were *Ent. faecalis* ATCC 19433 and *Ent. faecium* ATCC 19434, respectively (Table 4.1). Species D and E showed greatest similarity with *Ent. gallinarum* ATCC 49573 and *Ent. hirae* ATCC 8043, respectively, with similarity coefficients of > 0.90. Group B, F and G were most similar to *Ent. faecalis* ATCC 19433, *Ent. avium* ATCC 14025, and *Ent. dispar* ATCC 51266, respectively, with similarity coefficients of 0.70 to 0.80.

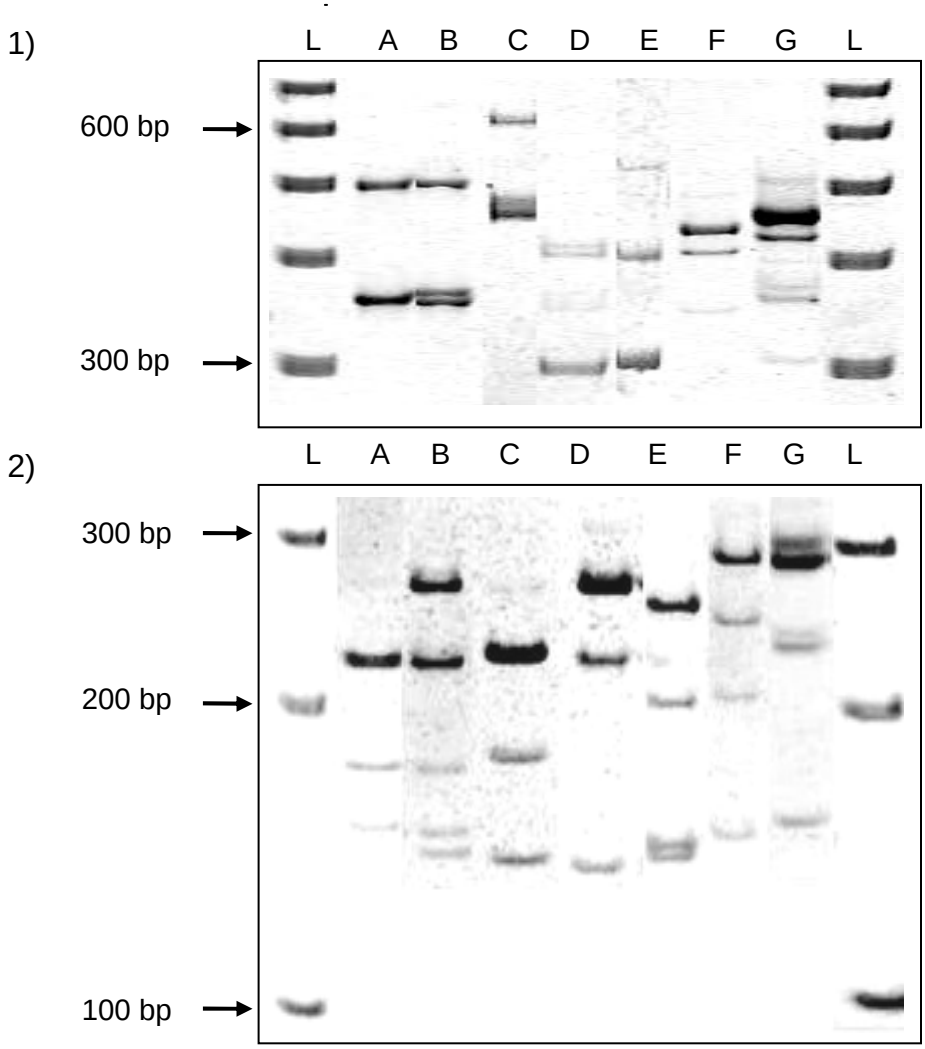

Figure 4.1. Banding patterns of representative isolates for the seven *Enterococci* groups as shown in 8% Polyacrylamide gel. These banding patterns were developed by PCR amplification of the intergenic spacer (ITS-PCR) between the 16S and 23S rRNA genes using primers ITS-RBS-L1, 5'-CAAGGCATCCACCGT-3', and ITS-RBS-G1, 5'-GAAGTCGTAACAAGG-3'. 1) The expected size of the amplicons varies from 300 to 600 bp and large minor bands. Often two major bands between 300 to 500 bp are observed. Primers were designed by Jensen et al. (1993) but applied to differentiate *Enterococcus* species by Tyrrell et al. (1997). 2) The PCR amplicons were subsequently digested with Sau3AI to obtain characteristic banding patterns that are species-specific. Dice similarity coefficients were taken to identify similarity with known ATCC standards. Seven groups of consistent banding patterns are observed among 702 *Enterococci* isolates. Lanes A to G are *Enterococci* groups, specifically: A – 1.0*, *Ent. faecalis,* B – 0.70*, *Ent. faecalis,* C – 1.0*, *Ent. faecium,* D – 0.90*, *Ent. gallinarum,* E – 0.97*, *Ent. hirae,* F – 0.80*, *Ent. avium,* and G – 0.80*, *Ent. dispar.* *Dice = 2C/ N1 + N2, where C is the no. of positive band matches, N1 is the total no. of bands in standard, and N2 is the total no. of bands in sample. L, 100 bp ladder.

Table 4.1. Dice Similarity Coefficient between known *Enterococci* standards and *Enterococci* isolates from water and sediment samples of eight sites in Stillwater Creek watershed.

Standards	*Enterococci* group of isolates							Molecular weight (bp) of common bands in the most similar species
	A	B	C	D	E	F	G	
Ent. faecalis ATCC 19433	1.0*	0.70*	0	0.30 [5]	0	0	0.30 [3]	220, 172, 147
Ent. faecium ATCC 19434	0	0.10 [1]	1.0*	0.30 [1]	0.39 [6]	0	0.30 [6]	227, 180, 142
Ent. gallinarum ATCC 49573	0	0	0.22 [1]	0.90*	0	0	0.20 [9]	297, 279, 220, 142
Ent. dispar ATCC 51266	0	0.14 [8]	0	0.30 [8]	0	0	0.80*	279, 227, 147
Ent. sulfureus ATCC 49903	0	0.20 [7]	0	0	0	0	0	n/a
Ent. avium ATCC 14025	0.3 [4]	0.20 [4]	0	0	0	0.80*	0	210, 144
Ent. hirae ATCC 8043	0	0	0	0	0.97*	0.30 [2]	0	254, 200, 144

1.0 = no distance, most similar species, 0 = greatest distance, least similar species. Dice Similarity Coefficient, Dice = 2C/ N1 + N2, where C is the no. of positive band matches, N1 is the total no. of bands in standard, and N2 is the total no. of bands in sample. Other bands found common in less related species were: [1] 142 bp, [2] 144 bp, [3] 147 bp, [4] 172 bp, [5] 220 bp, [6] 227 bp, [7] 276 bp, [8] 279 bp, and [9] 297 bp.

4.3.2. Distribution of *Enterococci* species in surface water and sediments

Of 702 isolates evaluated, 282 were group A *Ent. Faecalis,* and 198 were group B *Ent. Faecalis,* which took 68% of total isolates tested (Table 4.2). The remaining species were dominated by group C (*Ent. faecium*), followed by group D, E, F, and G. The distributions of *Enterococci* groups within each temporal/habitat condition were similar for most species detected, with one exception for group B *Ent. faecalis*. The percentage of this group of *Enterococci* in the sediment under baseflow condition was significantly lower than those detected in the surface water under both baseflow and highlow conditions.

The distribution of *Enterococci* species within each temporal/habitat condition at the eight sampling sites are shown in Figure 4.2. During baseflow conditions, Sites 1 and 4 had the least number of species (3 species) in the sediments, while Sites 3, 6 and 7 had the most number of species (5 species) in the sediments. Site 4 also had the most number of species (5 species) in the surface water during baseflow. During highflow conditions, Site 6 had the most number of species (6 species), while Site 8 had the least number of species.

Of the eight sites tested, group A was a dominant species in the surface water, composing about 59% of *Enterococci* detected within each site. Group A *Enterococci* was also found in the sediments of Sites 2, 5 and 4. Groups A, B, and C were found in the surface water and sediments of all sites during highflow and baseflow with mean percentages ranging from 6 to 59% except in Site 8

Table 4.2. Number of isolates (N) and percentage for each *Enterococci* group within the specified sampling condition and habitat.[1]

Temporal condition	Habitat		A - 1.0*, *Ent. faecalis*	B - 0.70*, *Ent. faecalis*	C - 0.70*, *Ent. faecium*	D - 0.90*, *Ent. gallinarum*	E - 0.97*, *Ent. hirae*	F - 0.80*, *Ent. avium*	G - 0.80*, *Ent dispar*	Total
High flow summer 2005 and 2008	Surface water	N	115	101	50	32	12	3	0	313
		%	36.7 [a]	32.2 [b]	16.0 [c]	10.2 [d]	3.8 [e]	1.0 [e]	0 [e]	
Base flow summer 2005 and 2008	Surface water	N	88	75	36	10	0	3	1	213
		%	41.3 [a]	35.2 [b]	16.7 [c]	4.7 [d]	0 [e]	1.4 [e]	0.5 [e]	
Base flow summer 2005 and 2008	Sediments	N	79	21	39	24	10	1	2	176
		%	44.9 [a]	11.9 [c]	22.2 [c]	13.6 [d]	5.7 [e]	0.6 [e]	1.1 [e]	
Total		N	282	197	125	66	22	7	3	702
		%	40.2 [a]	28.1 [b]	17.8 [c]	9.4 [d]	3.1 [e]	1.0 [e]	0.4 [e]	

Mean percentages followed by the same small letter in a row, and column are not significantly different from each other at 5% level of significance; three surface water and three sediments samples were collected from each of the eight sites in Stillwater Creek Watershed.

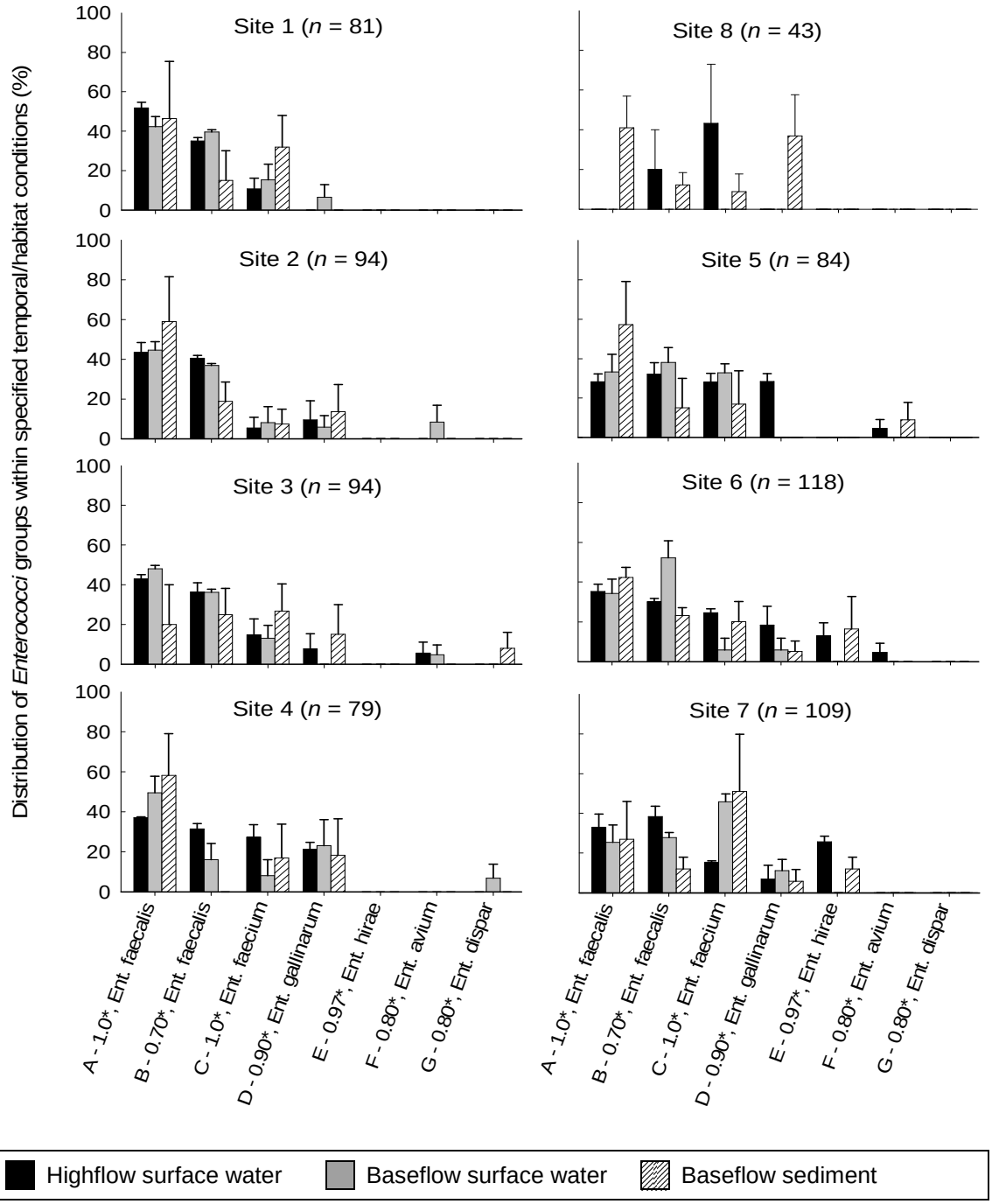

Figure 4.2. Distribution of *Enterococci* within a temporal/habitat conditions at eight study sites. Three conditions were evaluated, surface water during highflow conditions, and surface water and sediments during baseflow conditions. A-G, *Enteroccocci* diversity group as defined in Table 1. Bars indicate standard error and n indicates number of isolates tested.

where their occurrences varied. Group D was found in the surface water of all sites with mean percentages ranging from 7 to 29% during highflow except in Sites 1 and 8 where it was not found. Moreover, Group D was found in the surface water of all sites with means ranging from 6 to 23% during baseflow except in Sites 3, 5 and 8. It was also found in the sediments of all sites with means ranging from 5 to 37% during baseflow except in Sites 1 and 5. Furthermore, Groups E, F, and G were the least common species found at each site. Group E was found only in the surface water and sediments of Sites 6 and 7 with means ranging from approximately 12 to 26%. Group F was found in the surface water and sediments of Sites 3 and 5, and only in the surface water of Site 6 during highflow and Site 2 during baseflow. In addition, Group G was found only in the surface water of Site 4 and sediments of Site 3 during baseflow.

4.4. Discussion

Some *Enterococcus* sp. such as *Ent. faecalis* was reported to show host specificity rendering it as a potential fecal indicator for microbial source tracking (Molina, 2005; Kuntz et al., 2003; Rodgers et al., 2003; Wheeler et al., 2002). In this study, seven *Enterococcus* species groups were found to be distributed in the eight sampling sites with four found in all the sites. Temporal variability of each *Enterococci* species was limited in the surface water and sediments during baseflow and highflow periods, possibly because the isolates evaluated

represented only those collected during summer. Some reported *Enterococcus* species distribution to be highly variable on seasonal basis such as summer, fall, winter, or spring (Molina, 2005). The similar distribution of *Ent.* species in the surface water and sediments during baseflow and highflow conditions implies that these sites shared common sources of contamination or the species detected were commonly found in warm-blood animals with little host specificity. Within sites, there were some variations on the distribution of species when comparing surface water with sediments or baseflow with highflow. However, the general trend for occurrence of species was similar across sites evaluated, showing dominance of group A followed by group B. Three groups (Groups E, F, and G) showed some site specificity either in the surface water, sediments or in both during baseflow and highflow periods. The presence of group E in the creek water that passed through urbanization area suggests that this species may be closely related to human activities, consistent with the study conducted by Ferguson et al. (2005). On the other hand, the presence of group F in the creek water that passed through animal and crop production area as well as wildlife and recreation area suggests that this species can be contributed by animal sources. *Enterococcus avium* was found in cattle dung, dogs, and water samples impacted by urban activities (De Graef et al., 2005; Devriese et al., 1992a; Devriese et al., 1992b). The absence of this species in Sites 1, 4, 7 and 8 may indicate absence of its source at these sites or limited ability to survive and persist in the environment. Latter is evident by the low level detected (This

study; De Graef et al., 2005; Devriese et al., 1992a; Devriese et al., 1992b). The presence of group G in creek water that passed through agricultural production facilities can be contributed by domesticated agricultural animals. *Enterococcus dispar* is a recently described *Enterococcus* species (Collins et al., 1991) and studies on their occurrence in the environment was limited, although *Ent. dispar*-like microorganisms were reported in dogs (De Graef et al., 2005). However, this study suggests that *Ent. dispar* can be contributed by other animal sources, as well, because group G (closely similar to *Ent. dispar*) was found in areas impacted by agricultural production.

Group A matched 100% to the DNA profile of *Enterococcus faecalis* ATCC 19433, while group B matched 70%. This suggested that group B may be a different strain of *Ent. faecalis* or a subspecies. The occurrence of lower percentage of group B in the sediments indicates that it is less persistent in the environment than group A. *Enterococcus faecalis* commonly originates from fecal matters of humans or birds (Haack et al., 2009; Poucher et al., 1991), and of in dogs as well (De Graef et al., 2005; Devriese et al., 1992a). It is, therefore, not surprising that group A had the highest percentage (approximately 59%) in the sediments of Site 5 in this study. Site 5 was located within the vicinity of wildlife and recreation area, where there were abundant wildlife birds such as geese as well as a place popular for dog walking. *Enterococci faecalis* were suggested to be a potential indicator of fecal contamination from human sources (Wheeler et al., 2002; Poucher et al., 1991), however, the results of this study

suggests that *Ent. faecalis* found in Site 6 (site representing contamination from human sources) originate mainly from wildlife animals and dogs found in wildlife and recreation area located upstream of Site 5. This was suggested because the changes in percentage distribution of *Ent. faecalis* in Site 5 and Site 6 were limited. The same observation was found for group C suggesting that this species was contributed mainly by animals rather than humans regardless of whether animals are domesticated or wild. Human contribution of *Ent. faecium* in this watershed is limited although this species had been isolated from humans sources (Haack et al., 2009). It is not surprising to observe *Ent. faecium* in areas impacted by animals because it was found dominant, as well, in animal sources such as cattle and dogs (De Graef et al., 2005; Molina, 2005; Laukova et al., 1998).

The distribution of group D indicates possible contribution of human sources to fecal contamination in the watershed because group D was found during three temporal conditions in Site 6. However, it is unclear whether it is mainly contributed by human sources because it was found in the surface water of Site 5 during highflow, and sediments of Site 8. It was also found during three temporal conditions in Site 2 implying that the source of contamination is located upstream of this site which was not evaluated in this study. A study conducted by Wheeler et al. (2002) showed that *Ent. gallinarum* was isolated from wild deer at high percentage of up to 66%. However, in this study, no site represented sources such as deer. It is thus suggested that this species (Group D) had

sources other than humans and was transported by water run-off during heavy rain and rapid water discharge, as explained by their occurrence during highflow condition (Jeng et al., 2005; Crump et a., 1998).

Although it was suggested in some studies that *Enterococcus* sp. such as *Ent. faecalis* shows host specificity rendering it as potential fecal indicator for microbial source tracking (Molina, 2005; Kuntz et al., 2003; Rodgers et al., 2003; Wheeler et al., 2002), this study suggests otherwise because *Ent. faecalis, Ent. faecium* and *Ent. gallinarum* were found common and dominant in all the sites. On the other hand, the less dominant species, *Ent. avium, Ent. hirae* and *Ent. dispar*, composed of 0.4 to 3.1% of the detected *Enterococci*, may be more useful in microbial source tracking.

4.5. Conclusion

Seven *Entercococci* groups representing six species were found in the surface water and sediments during baseflow and highflow conditions, including *Ent. faecalis*, *Ent. faecium, Ent. gallinarum, Ent. hirae, Ent. avium* and *Ent. dispar*. *Enterococci faecalis* was the most dominant species of 702 isolates tested and at seven out of eight sampling sites evaluated. The less dominant species, composed of 0.4 to 3.1% of the detected *Enterococci*, may be more useful in microbial source tracking.

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CHAPTER V

SUMMARY AND CONCLUSION

Enterococci were detected and quantified in the surface water and sediments at eight locations under four temporal conditions including baseflow June 2005, baseflow July 2008, baseflow November 2008, and highflow May 2008. The locations were selected using targeted sampling strategy to examine three potential fecal microbial sources, including intense animal production, urbanization, and intense wildlife and recreation activities. Seven hundred two (702) *Enterococci* isolates obtained were evaluated for diversity and distribution in the Stillwater Creek Watershed through analysis of DNA banding patterns that were developed by PCR amplification of the intergenic spacer (ITS-PCR) between the 16S and 23S rRNA genes. The banding patterns were compared using Dice similarity coefficient in reference to ATCC *Enterococci* cultures.

With the exception of one site, *Enterococci* concentrations exceeded the USEPA recommended level in 162 samples tested. Temporal variability depended on the flow conditions rather than the sampling season.

Concentrations of *Enterococci* in the surface water during highflow period were hundreds times of those sampled during baseflow period. Therefore, effective stormwater management is critical in maintaining water quality in the environment. Not only was highflow a significant source of *Enterococci* to the water environments, but also considerable *Enterococci* persisted in the sediment, making sediment a reservoir of *Enterococci* and potential source of contamination.

Of 702 *Enterococci* evaluated, seven groups were revealed with 4 groups (Groups A, B, C, and D) common to all sites and three (Groups E, F, and G) showing some site specificity. These species groups represented six *Enterococcus* species including *Ent. faecalis* (Groups A and B), *Ent. faecium* (Group C), *Ent. gallinarum* (Group D), *Ent. hirae* (Group E), *Ent. avium* (Group F) and *Ent. dispar* (Group G). *Enterococcus feacalis* was the most dominant species composed of 68.3% of the 702 isolates evaluated. Although some studies suggested that *Enterococcus* sp. such as *Ent. faecalis* shows host specificity rendering it an ideal indicator for microbial source tracking, this study suggests otherwise; the less dominant species in the watershed including *Ent. avium, Ent. hirae* and *Ent. dispar*, may be more useful in microbial source tracking.

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Scope and Method of Study: *Enterococci* in creeks were evaluated with respect to temporal and spatial variation, potential sources, abundance, and diversity in surface water and sediments at eight locations under four temporal conditions.

Findings and Conclusions: With the exception of one location, *Enterococci* concentrations exceeded the USEPA recommended level for impaired watershed, suggesting the crucial role of creek water in governing water quality in the environment. Moreover, effective storm water management is critical in maintaining water quality because concentrations of *Enterococci* in the surface water during highflow were approximately 100 times of those sampled during baseflow. Sediment could serve as a reservoir of *Enterococci* for the water system, evidenced by their high levels in sediments. Of 702 isolates evaluated, six *Enterococcus* species were found with *Ent. feacalis* being most dominant. The less dominant species such as *Ent. hirae*, *Ent. avium*, and *Ent. dispar* may be more useful in microbial source tracking for the watershed evaluated.

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